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PRINCIPAL INVESTIGATOR: Glen Kisby, Ph.D.

CONTRACTING ORGANIZATION: Center for Oregon Health Sciences University
Portland, Oregon 97201-3098

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Glen Kisby, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Center for Oregon Health Sciences University
Portland, Oregon 97201-3098

E-MAIL:
kisby@ohsu.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Animal colonies were developed from breeder pairs of mice heterozygous for the DNA repair genes O^6-methylguanine methyltransferase (*Mgmt*), 3-methyladenine DNA glycosylase (*Aag*), and xeroderma pigmentosum group A (*Xpa*). Null *Mgmt*-, *Xpa*-, and *Aag*-mice were found to be fertile indicating that separate wild-type, heterozygous and homozygous neuronal and astrocyte cell cultures could be successfully prepared from each genotype (+/-; -/-). Cerebellar neuronal cultures of DNA repair-deficient (i.e., *Xpa* -/-) and *Mgmt*-overexpressing ($Mgmt^+$) mutant mice were examined for sensitivity to nitrogen mustard (HN2) and methylazoxymethanol (MAM). *Xpa* (-/-) neuronal cultures were ~2-3x more sensitive than *Xpa* (+/-) neurons to low concentrations of HN2 (0.5 µM to 5.0 µM). *Xpa* (-/-) neurons responded in a similar manner after a brief exposure to low-dose UV irradiation. In contrast, neurons that overexpressed $Mgmt^+$ were protected from short-term (24 h) exposure to high concentrations of MAM (100 µM), but were equally sensitive to HN2 (0.1 µM, 1.0 µM). Results from these preliminary studies suggest that modulation of DNA repair can alter the sensitivity of neurons to HN2 or other genotoxins. Additional studies are underway to confirm these findings and to determine whether these effects occur in similarly treated astrocytes of mice with altered DNA repair.

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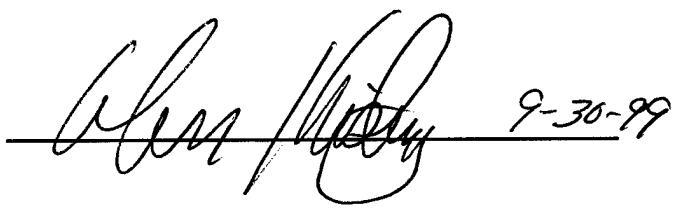

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INTRODUCTION

Experiments are proposed to examine the molecular mechanism by which mustard chemical warfare agents induce neuronal cell death. DNA damage is the proposed underlying mechanism of mustard-induced neuronal cell death. We propose a novel research strategy to test this hypothesis by using mice with perturbed DNA repair to explore the relationship between mustard-induced DNA damage and neuronal cell death. Initial *in vitro* studies (Years 1, 2 & 3) are proposed to examine the cytotoxic and DNA damaging properties of the sulfur mustard analogue mechlorethamine (nitrogen mustard or HN2) and the neurotoxic DNA-damaging agent methylazoxymethanol (MAM) using neuronal and astrocyte cell cultures from different brain regions of mice with perturbed DNA repair. Findings from these studies will be used to examine the *in vivo* neurotoxic effects of HN2 and MAM (Years 3 & 4) in mice with perturbed DNA repair.

BODY OF THE REPORT

STATEMENT OF WORK FOR YEAR 1 of FUNDING

The overall goal of studies proposed in Year 1 are to: (i) obtain DNA repair-deficient mice, (ii) develop colonies of wild-type and DNA repair-deficient mice, and (iii) develop neuronal and astrocyte cell cultures of wild-type and DNA repair-deficient mice. Specific objectives proposed in Year 1 of the Statement of Work are as follows:

1. Obtain *Aag*- and *Mgmt*-deficient mice from L. Samson and establish colonies.
2. Expand current colony (2 breeder pairs) of ERCC1-deficient mice.
3. Develop neuronal and astrocyte cell cultures from C57BL/6 mice (*wild-type*).
4. Examine cytotoxicity of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.
5. Develop neuronal and astrocyte cultures from Aag-, ERCC1- and MGMT-deficient mice.

In general, a majority of these research objectives were achieved during the first year of funding. The simultaneous establishment of 1-wild type and 3-DNA repair deficient colonies precluded us from developing both neuronal and astrocyte cell cultures. Therefore, studies in Year 1 were focused on establishing neuronal cultures from wild type and DNA repair-deficient mice. A detailed description of the research accomplishments for each objective of Year 1 follows.

1. Obtain *Aag*- and *Mgmt*-deficient mice from L. Samson and establish colonies.

Heterozygous mice with *Aag* and *Mgmt* knockout mutations were obtained from L. Samson (Harvard University, Boston MA) and heterozygous mice with *Xpa* deficiency were obtained from K. Tanaka (Osaka University, Japan). Heterozygous crosses were set up to obtain mice with both heterozygous and homozygous deficiencies. PCR conditions were established for all genotypes, though at the present time primers specific for the *Aag* and *Mgmt* knockout mutations have not been used successfully. (Members of Dr. Samson's laboratory have also reported similar problems to us). At the present time we are using primers that are specific for the *neo* insertions to identify mice with the knockout mutations. We tested and successfully demonstrated that males and females homozygous for *Aag*, *Mgmt*, and *Xpa* are fertile. These observations will allow us to create litters with specific genotypes, i.e. wild-type, heterozygous-deficient, and homozygous-deficient (*see also #5*). The ability to generate litters of a specific genotype is especially important for the successful development of neuronal and astrocyte cell cultures from embryonic nervous tissue (E14-15) of DNA repair-deficient mice.

In addition to the current colonies of DNA repair-deficient mice, brain tissue was also obtained from a transgenic mouse mutant that overexpresses O^6-methylguanine methyltransferase (MGMT; S. Gerson, Univ. Cincinnati). This mouse will be used to determine if overexpression of MGMT protects neurons against HN2- or MAM-induced cytotoxicity. The *Mgmt*$^+$ mouse mutant developed by Dr. Gerson was chosen over that of another mutant mouse (C. Walter, Univ. Texas) [11] because his mouse mutant (unlike Dr. Walter's) overexpresses MGMT in neurons [3]. Moreover, DNA repair activity is reportedly 15x higher in the brain of this *Mgmt*$^+$ mutant mouse when compared with brain tissue of wild-type mice (S. Gerson, *personal communication*). Because of the limited space and funds for creating another colony of mutant mice, a novel method was developed to prepare viable cerebellar neuronal and astrocyte cell cultures from the brains of MGMT$^+$ mutant mice shipped to us on ice [based upon the protocols of Brewer *et. al.* [1]]. Briefly, brains from litters containing wild-type and MGMT$^+$ mice were removed (by members of Dr. Gerson's laboratory), the tissue immediately placed in conical tubes containing 2.0 ml sterile Hibernate/B27 media (GibcoBRL) and the brain tissue shipped overnight on ice. Several months of culturing were required before viable (>50%) cerebellar neuronal cultures could be prepared from brain tissue sent to us by Dr. Gerson's laboratory (see experiment 5-3-99; **Figure 2**, Appendix). A major reason for the poor viability of MGMT$^+$ neurons was the inexperience of the members of Dr. Gerson's laboratory with the dissection of nervous tissue from mice. Reducing the time of dissection, replacement of expired Hibernate/B27 with fresh media following the shipment of brain tissue, and increasing the concentration of horse serum (15%) in the cell culture media were improvements that were made to our cell culture protocol to increase cell viability. With these new protocols viability of cerebellar neurons improved to >90% after plating and ~ 60-80% prior to genotoxin exposure (7 days *in vitro*) (see experiment 6-22-99, **Figure 2**, Appendix),. This method has also been used to obtain cerebellar neuronal cultures from brain tissue of wild-type or heterozygous AP endonuclease (APE)-deficient mice (shipped overnight on ice) with comparable viability [8](GK, *unpublished data*).

2. Expand current colony (2 breeder pairs) of ERCC1-deficient mice.

We attempted to develop a colony of ERCC1-deficient mice, but several months of breeding failed to generate sufficient numbers of 'null' (homozygous) mice for expansion of the colony. These findings are supported by recent studies which demonstrate that breeding of ERCC1 heterozygotes results in reduced frequency of the homozygous mutant mouse [6]. More importantly, both female and male mice homozygous for ERCC1 are infertile [12], making this strain unsuitable for preparing separate neuronal and astrocyte cultures of each genotype (i.e., heterozygote, homozygote). Consequently, we contacted Dr. K. Tanaka (Osaka, Japan) to obtain his *Xpa* mutant mouse, a nucleotide excision repair (NER)-deficient mouse in which the homozygous mutant is fertile [*see also* #1]. Breeder pairs of mice heterozygous for *Xpa* were obtained from Dr. Tanaka, mated and colonies established for these mice. The *Xpa* gene codes for a NER protein that is responsible for recognizing bulky adducts and x-links [2,10]. Neuronal cells prepared from *Xpa*$^{-/-}$ mice (like ERCC1-deficient mice) should be incapable of removing HN2-induced x-links and be particularly sensitive to HN2. More importantly, Dr. Tanaka has recently shown that cerebellar neuronal cultures prepared from his *Xpa*$^{-/-}$ mutant mouse are hypersensitive to UV irradiation [4]. These findings are strong evidence that the *Xpa*-deficient mouse is a more suitable strain (*vs.* ERCC1 mutant mice) to examine the role of x-links in HN2-induced neuronal cell death.

3. Develop neuronal and astrocyte cell cultures from C57BL/6 mice (*wild-type*).

The colony of wild-type mice that was established during the 1st year of study was used primarily to develop colonies of *Aag*-, *Mgmt*-, and *Xpa*-mutant mice and train technical staff in the methods for developing cultures of cerebellar neurons and astrocytes. Of the few litters available for training, none

of the cerebellar neuronal cultures were sufficiently viable (>50%; fluorescence assay, *see #4 for details*) to assess the cytotoxicity of HN2 or MAM. In contrast, cerebellar neuronal cultures were successfully developed from litters that contained both wild-type and *Mgmt*$^+$ mice. After several months of culturing (see #1 for details), we were able to achieve a viability of ~60-80% for cerebellar neuronal cultures. Recent improvements in our cell culture protocol (e.g., concentration of FBS, increased cell density) have increased the viability of rat cerebellar neuronal cultures (~90%) and will be used to attain similar viability of mouse cerebellar neuronal cultures. Some of these improvements have been instituted within the past several months and the viability of cerebellar neurons from wild-type and *Mgmt*$^+$ mice brain tissue has increased ~2x [see **Figure 1,** *Appendix*].

4. Examine cytotoxicity of HN2 and MAM in C57BL/6 neuronal and astrocyte cell cultures.
The cytotoxic properties of HN2 and MAM was examined in neuronal cultures prepared from wild-type mice obtained from crosses of *Mgmt*$^+$ heterozygotes (C57BL/6 background)(see #1 for details). Wild-type neuronal cultures were treated for 24 h with control culture media or culture media containing various concentrations of HN2 (0.1 μM, 1.0 μM) or methylazoxymethanol (MAM; 50 μM, 100 μM) [**Figure 2,** *Appendix*]. For comparison, neuronal cultures were prepared from *Mgmt*$^+$ littermates and treated in a similar manner with HN2 and MAM. These concentrations of HN2 or MAM have been shown to produce cell death of cerebellar [9] or cortical neurons [5], respectively. Cell viability was determined by manually counting the number of fluorescently labeled cells (calcein-AM and prodium iodide) as previously described by Kisby *et al.* [9]. Wild-type and *Mgmt*$^+$ neuronal cultures were incubated for 30 min with the fluorochromes calcein-AM and prodium iodide (PI), the cell culture media removed, and the cell monolayer examined by fluorescence microscopy for cell density. Cell density was determined by taking fluorescent photomicrographs of neurons from 2 fields (~500-1000 cells/field), the 35 mm slides scanned and the numbers of calcein-AM (green fluorescence) and PI (red fluorescence) cells counted manually from each micrograph [9].

The viability of untreated wild-type and *Mgmt*$^+$ cerebellar neurons was ~60-75%. Neuronal survival after exposure to 50 μM MAM for 24 h was similar for wild-type and *Mgmt*$^+$ cells. However, *Mgmt*$^+$ cerebellar neurons were noticeably resistant to higher concentrations of MAM (100 μM) while wild-type cells became sensitive. In contrast, wild-type and *Mgmt*$^+$ cerebellar neurons appeared to be equally sensitive to HN2 treatment. Additional studies using lower concentrations of HN2 are underway to confirm these findings. These findings suggest that MGMT protects neurons from MAM-induced cytotoxicity, but not from HN2-induced cytotoxicity (which produces predominantly N^7-alkyl DNA adducts). The increased resistance of *Mgmt*$^+$ neurons to high concentrations of MAM may be explained by the elevated repair of O^6-methylguanine DNA adducts, a lesion that may be responsible for MAM-induced neuronal cell death. *Mgmt*$^+$ neurons were not protected from HN2-induced cytotoxicity because this genotoxin produces predominantly N^7-alkyl DNA adducts and x-links, adducts not repaired by MGMT. Additional studies are underway to confirm these findings.

5. Develop neuronal and astrocyte cultures from Aag-, ERCC1- and MGMT-deficient mice.
During the 1st year of the grant, we prepared neuronal cell cultures from the cerebellum of *Mgmt*-/-, *Mgmt*$^+$, *Xpa*$^{+/-}$, and *Xpa*$^{-/-}$ mice. *Xpa* mutant mice were used in place of ERCC-1 mice for generating neuronal and astrocyte cell cultures that are deficient in NER (for details see #1). *Mgmt*$^{-/-}$ cerebellar neuronal cultures (7 days *in vitro*) were treated with various concentrations of MAM (0.1 μM, 1.0 μM, 10 μM, 1000 μM) and HN2 (0.01 μM, 0.1 μM, 1.0 μM, 5.0 μM) or UV irradiation (9.6 J/m^2). Unfortunately, technical problems with our fluorescent microscope prevented us from assessing the viability of the fluorescently labeled cells. However, we were successful in examining the cytotoxicity of HN2 and MAM in cerebellar neurons from *Mgmt*$^+$, *Xpa*$^{+/-}$, and *Xpa*$^{-/-}$ mutant mice. Cytotoxicity was

determined by counting the number of viable neurons or the leakage of LDH into the cell culture media [7]. Neuronal cell cultures developed from *Mgmt*$^{+}$ mutant mice were grown for 7 days *in vitro* treated for 24h with various concentrations of nitrogen mustard (HN2; 0.1 μM, 1.0 μM) or methylazoxymethanol (MAM; 50 μM, 100 μM) and examined for cell viability by manually counting the number of fluorescently labeled cells (see #4 for details) [**Figure 2,** *Appendix*]. Neuronal cultures developed from *Xpa*$^{+/-}$ and *Xpa*$^{-/-}$ mutant mice were also grown for 7 days *in vitro*, treated for 24h with HN2 or UV irradiation and viability determined by measuring LDH activity in culture media of treated cells as previously described by Kisby *et al.* [7] [**Figure 3,** *Appendix*].

Cerebellar neurons that overexpress *Mgmt*$^{+}$ were protected from high concentrations of MAM, but appeared equally sensitive to HN2-induced cell death (see #4 for details). In contrast, LDH activity (an indicator of loss of membrane integrity) was ~1.5-3.0x higher in HN2-treated *Xpa*$^{-/-}$ deficient cerebellar neurons than comparably treated *Xpa*$^{+/-}$ neurons. LDH activity was also ~3.0x higher in low-dose UV-irradiated *Xpa*$^{-/-}$ deficient cerebellar neurons than comparably treated *Xpa*$^{+/-}$ neurons. The similar sensitivity of *Xpa*$^{-/-}$ deficient cerebellar neurons to HN2 and UV irradiation suggests that x-links may play an important role in HN2-induced cell death. Additional studies with *Xpa*-, *Aag*- and *Mgmt*-deficient cerebellar neuronal cultures will be required to determine the significance of these findings.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully created litters with specific genotypes (i.e., wild, heterozygous, homozygous).
- Obtained DNA repair-overexpressing mice (i.e., *Mgmt*) for protection studies and developed a method to culture viable neurons and astrocytes from brain tissue of these animals.
- Developed viable neuronal cell cultures from wild-type and DNA repair-deficient mice.
- Demonstrated that neurons from nucleotide excision repair (NER)-deficient mice (i.e., *Xpa*) are hypersensitive to HN2 and UV irradiation.
- Demonstrated that neurons overexpressing *Mgmt* are protected from MAM-induced toxicity.

REPORTABLE OUTCOMES

Findings from the first year studies were presented at the 33rd Annual Winter Conference on Brain Research (WCBR) at Breckinridge, CO (January 22-29, 2000). A panel of speakers (S. LeDoux, G. Kisby, P. Brooks, T. Nouspikel) have been invited by Dr. P. Hanawalt (Stanford University) to speak on "DNA Repair in the Nervous System".

CONCLUSIONS

The primary objective of the 1st year of study was to obtain DNA repair-deficient mice and establish animal colonies. Once established, animals from these colonies could be used to develop neuronal and astrocyte cell cultures. We successfully obtained all of the DNA repair-deficient mice (*Mgmt*, *Aag*) and established colonies of these animals as proposed in the Statement of Work. Because of problems encountered during the development of a colony of ERCC1 knockout mice, another mouse strain was obtained (i.e., *Xpa* knockout) and a colony established. Like the ERCC1 knockout mouse, the *Xpa* knockout mouse is deficient in NER and the protein encoded by *Xpa* repairs bulky adducts and x-links [2,10]. More importantly, neurons from these mice have already been shown to be hypersensitive to UV irradiation [4], an effect we observed in similarly treated cerebellar neurons of our *Xpa*$^{-/-}$ mice.

The development of neuronal and astrocyte cell cultures from DNA repair-deficient mice for cytotoxicity studies was another objective of the 1st year of study. The problems encountered during the establishment of DNA repair-deficient and wild-type colonies and the establishment of litters with specific genotypes (i.e., heterozygous, homozygous) prevented us from developing both neuronal and astrocyte cell cultures. Consequently, we focused our attention on developing neuronal cultures from DNA repair-deficient mice because of the considerable effort required to develop viable neuronal cultures (*vs.* astrocytes). Neuronal cultures were developed from the cerebellum of *Mgmt*$^{-/-}$, *Xpa*$^{-/-}$, *Xpa*$^{+/-}$ and *Mgmt*$^{+}$ mice and examined for their sensitivity to HN2, MAM or UV irradiation. Preliminary studies with *Mgmt*$^{+}$ cerebellar neurons indicate they are protected from MAM-, but not HN2-induced cytotoxicity. In separate experiments, we demonstrated that *Xpa*$^{-/-}$ cerebellar neurons are more sensitive than *Xpa*$^{+/-}$ neurons to both HN2 and low-dose UV irradiation. Cerebellar neurons from both *Xpa*$^{-/-}$ and *Xpa*$^{+/-}$ mice have been previously shown to respond in a similar manner to UV irradiation [4]. These preliminary findings suggest that survival of cerebellar neurons depends on the repair of HN2- and MAM-induced DNA damage. Additional studies are required with neuronal and astrocyte cell cultures of *Xpa*$^{-/-}$ and other DNA repair-deficient mice to determine the significance of these findings with respect to DNA damage and neuronal cell death.

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APPENDIX

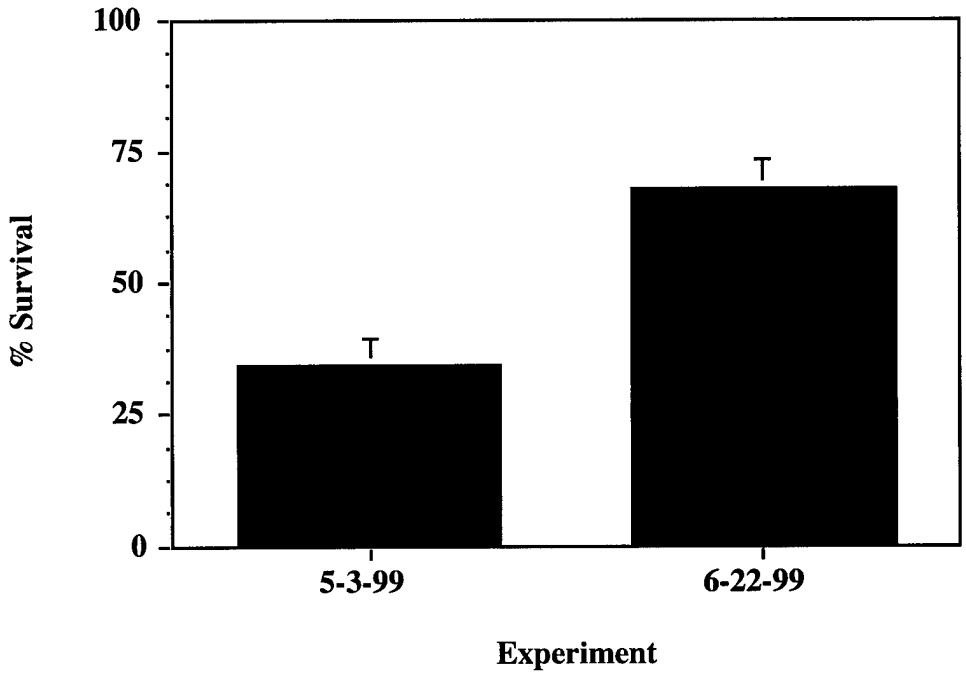

Figure 1. Viability of cerebellar neurons from brain tissue of wild-type mice shipped overnight on ice. Cerebellar granule cell neurons from two different experiments were cultured for 7 days *in vitro* and the cultures incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide). Cell viability was assessed by counting the total number of live (*green*) and dead (*red*) cells of fluorescent photomicrographs taken from 2 random fields (~500-1000 cells/field) of each well, as previously described by Kisby *et al.* [9]. Values represent the mean ± SEM (*n*= *4*).

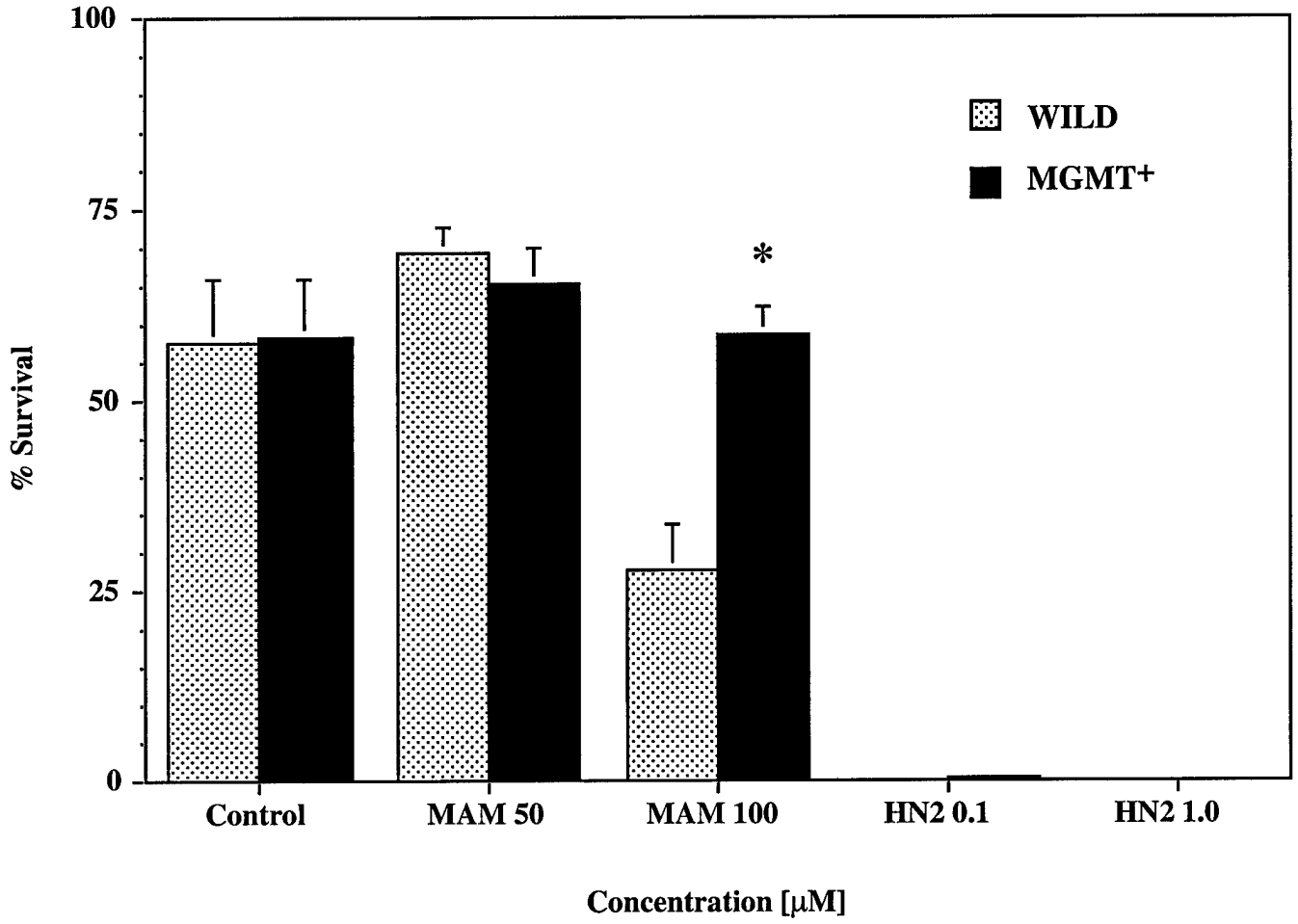

Figure 2. Viability of HN2 and MAM treated cerebellar neurons from wild-type (WILD) and *Mgmt*-overexpressing ($MGMT^+$) mice. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM, 1.0 μM) or MAM (50 μM, 100 μM) for 24 h, the culture media removed and the cultures incubated with fluorochrome containing culture media (0.26 μM calcein-AM and 3.0 μM propidium iodide). Cell viability was assessed by counting the total number of live (*green*) and dead (*red*) cells of fluorescent photomicrographs taken from 2 random fields (~500-1000 cells/field) of each well, as previously described by Kisby *et al.* [9]. Values represent the mean ± SEM ($n= 4$). *Significantly different from 100 μM MAM treated wild-type cells ($p < 0.05$, ANOVA).

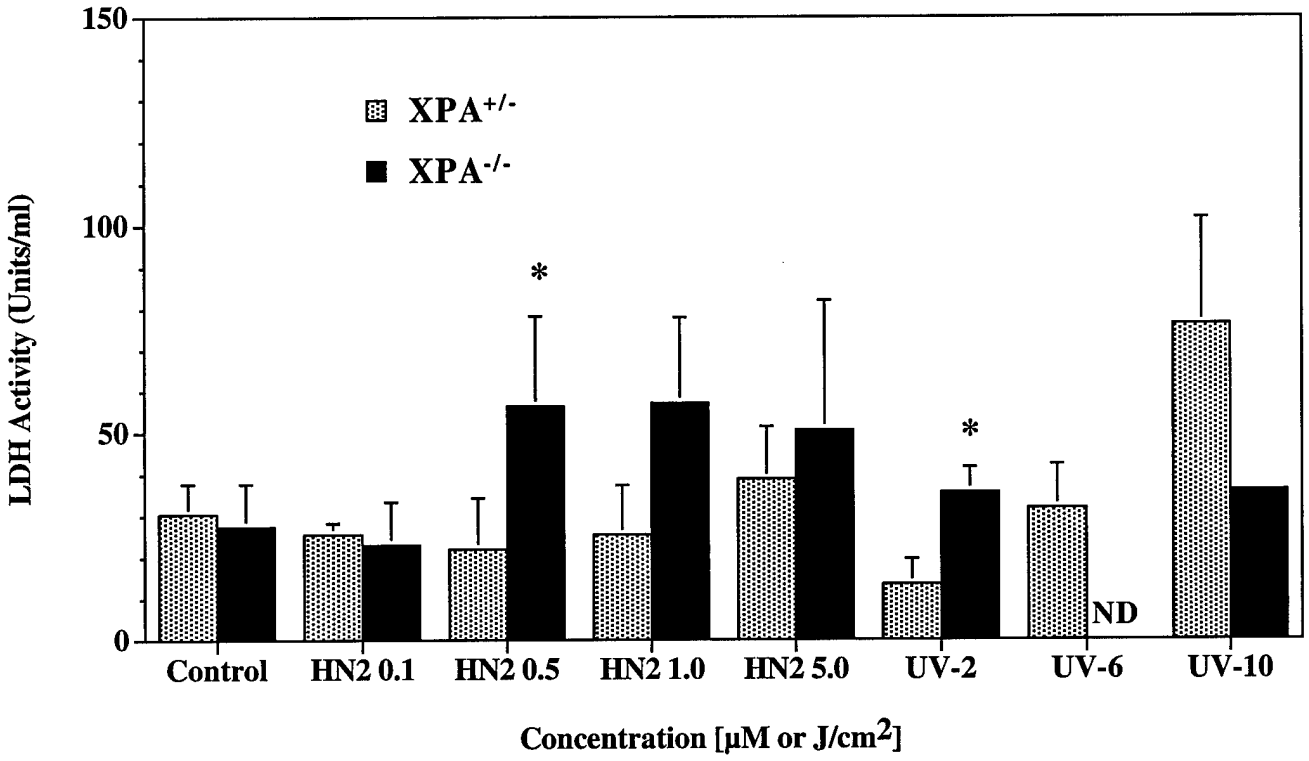

Figure 3. Lactate dehydrogenase (LDH) release from HN2 and MAM treated cerebellar neurons of mice heterozygous ($XPA^{+/-}$) or homozygous ($XPA^{-/-}$) for XPA. Mouse cerebellar granule cell cultures were treated with various concentrations of HN2 (0.1 μM, 0.5 μM, 1.0 μM, 5.0 μM) for 24 h or UV irradiation (2 J/cm^2, 6 J/cm^2, 10 J/cm^2), the cell culture media removed and assayed for LDH activity as previously described by Kisby *et al.* [9]. Values represent the mean ± SEM (n= *4*). ND = not determined. * Significantly different from 0.5 μM HN2-treated or UV-irradiated $XPA^{-/-}$ cells ($p<0.05$, ANOVA).